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Gene detection and expression profiling of *Neisseria meningitidis* using NanoString nCounter platform

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Abstract

Detection of bacterial gene transcripts in low density mucosal samples is challenging. We evaluated the NanoString nCounter system for transcript detection in *Neisseria meningitidis* (Nm) cultures. The method was sensitive, reproducible (R^2=0.99) and demonstrated changes in gene expression. Studying Nm transcripts from pharyngeal samples may be feasible using this approach.

Text

Neisseria meningitidis (Nm), the meningococcus, is a common commensal of the human pharynx that occasionally causes invasive disease. Primary prevention of meningococcal disease has relied, until recently, on polysaccharide conjugate vaccines. Novel protein-antigen vaccines, Bexsero®, containing four main antigens fHbp, NadA, NHBA, and PorA (outer membrane vesicle), and Trumenba®, containing two fHbp variants have now become available. Detecting transcripts of established and candidate Nm vaccine proteins from pharyngeal samples of Nm carriers may help predict their potential value in inducing mucosal immune responses that could reduce carriage and transmission.

Establishing an effective gene expression profiling platform in upper respiratory tract mucosal samples is particularly challenging due to the presence of both host mucosal cells and multiple bacterial species in the nasopharynx and because Nm is only present at low density in most pharyngeal carriers (1). The NanoString nCounter digital multiplexed gene detection and counting system has been shown to detect and quantify messenger RNA (mRNA) from low quantity samples accurately (2, 3). This method has previously been used to quantify mRNA transcripts directly in samples containing mixed bacteria using species-specific nucleotides without the need for bacterial isolation, complementary DNA synthesis or amplification (4-6). We evaluated the performance of this system in detection and quantification of Nm mRNA transcripts in *in vitro* cultures of Nm.

For a panel of 47 selected Nm genes, plus three housekeeping reference genes included for data normalisation, a codeset of 100 base length probes complementary to the gene target sequences was designed by NanoString, Seattle, Washington. Fifty bases were attached to a

biotin molecule, the capture probe, and the other 50 bases made up a reporter probe attached to colour coded fluorescent barcode specific to each gene assayed. One reporter count is equal to one transcript. The probes were designed to detect multiple isoforms of the selected Nm genes by targeting the conserved region of the genes, including those with known phase variability. They were not designed to identify the different variants of a gene. Genes were included that encode proteins in the meningococcal protein vaccines(7), candidate vaccine antigens identified from a literature search and proteins potentially involved in colonisation based on predicted surface expression (8-12). We included one gene likely to be involved in heat stress response, namely *dsbA-2.*

Experiments were set up to measure differences in gene expression levels following incubation at different temperatures. A standard ATCC-BAA-335 Nm strain was cultured on Colombia blood agar (Oxoid) at 37**°**C for 16 hours in 5% CO_2. Colonies were suspended in phosphate-buffered saline (PBS) with calcium and magnesium ions to OD_{600} 1 and inoculated in 1:100 dilution into three tubes each containing 10ml brain heart infusion supplemented with 2% isovitalex (BD BBL^{TM}) and 10% heat inactivated horse blood (TCS Biosciences). At mid-logarithmic phase (OD_{600}0.5/ ~8x10^{8}cfu/ml), the liquid cultures were each split into three equal volumes and exposed to cold shock 26**°**C, heat shock 40**°**C or held at 37**°**C (as controls) for three hours, centrifuged at 5,000*g* for 2 minutes and re-suspended in 20µl RNase free PBS (PAA Laboratories). RNAlater was immediately added to the suspension, stored at 4**°**C overnight, and then frozen at -80**°**C.

Prior to RNA extraction, samples were thawed on ice, diluted with an equal volume of ice cold PBS and centrifuged at 5000*g* for two minutes. Bacterial pellets were lysed with 15mg/ml lysozyme (Sigma) plus Tris-EDTA buffer, pH 8 for 30 minutes at room temperature in the presence of proteinase K (Sigma). Bacterial RNA was extracted using RNeasy mini kit following the manufacturer's protocol; on-column DNase digestion was performed using RNase-free DNase set (Qiagen). RNA quality, quantity and integrity were assessed using the Nanodrop Denovix D-11+ Spectrophotometer and Agilent 2200 Tape station system.

Aliquots of the extracts containing 100 ng/µl total RNA were run on the NanoString nCounter system. For the temperature experiments, one aliquot was taken from each of the three heat and cold shock samples, and two from each of the three 37**°**C control samples. In addition,

to assess reproducibility and the lower limit of detection, 37°C samples were split into two technical replicates then diluted 4-fold to run samples of 100, 25, 6.25,1.56, 0.4, and 0.1 ng/µl total RNA. Two aliquots were run for each of the diluted samples for each replicate. Six positive and eight negative hybridisation controls were pre-mixed with the codeset during manufacturing. The assay followed the XT gene expression protocol (NanoString). A 5 µl sample of the extracted RNA sample was hybridised with a custom designed probeset at 65°C for 19 hours in a thermocycler. Post-hybridisation purification and digital readouts of the gene count were done using the automated prep-station and digital analyser. Assay quality control metrics such as field of view count, binding density and positive control linearity were assessed before analysis. Except for the serial dilution samples, the data were normalised using positive controls and the housekeeping genes on nSolver 3.0 software. The internal positive controls were synthetic primers designed by the external RNA control consortium (ERCC) to not be homologous to any known organism. They were pre-mixed into the codeset. Six positive controls were included in every assay. Housekeeping gene normalisation was done for heat and cold shock experiments to adjust gene counts relative to expression of three housekeeping genes (*abcZ*, *adk* and *aroE*) according to the NanoString data analysis guide (13).

Statistical analysis was performed using Stata14 software. Multilevel mixed-effects linear regression was used to assess the relationship between gene expression and the different temperature conditions for the four vaccine genes, allowing for the multiple control samples.

Serial dilution of RNA preparations demonstrated that gene expression signals could be detected from total RNA concentrations as low as 0.1 ng/µl (Table S1). There was a high degree of reproducibility between the means of the duplicates for the two technical replicates (Figure 1, R^2=0.99).

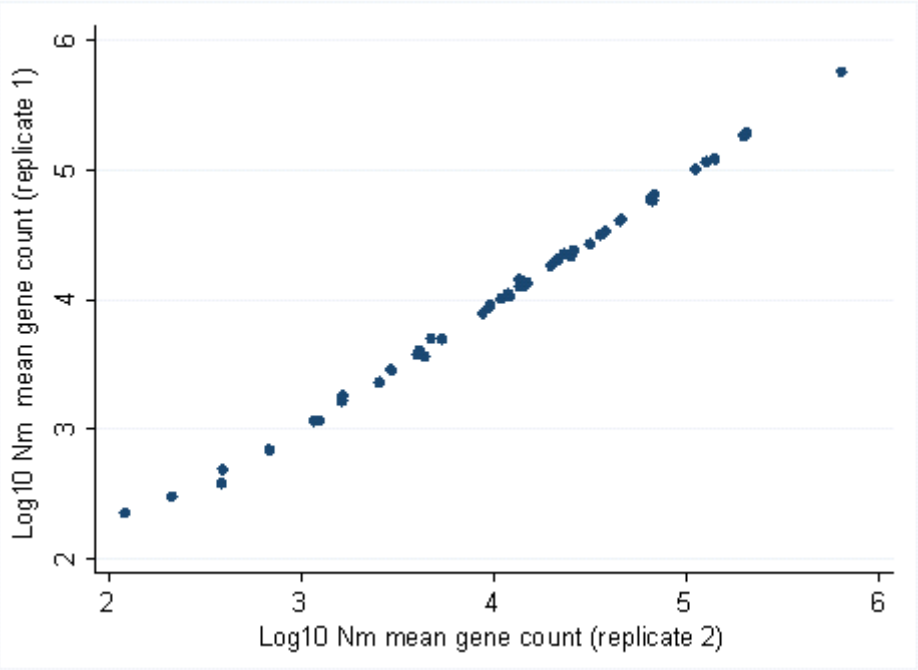
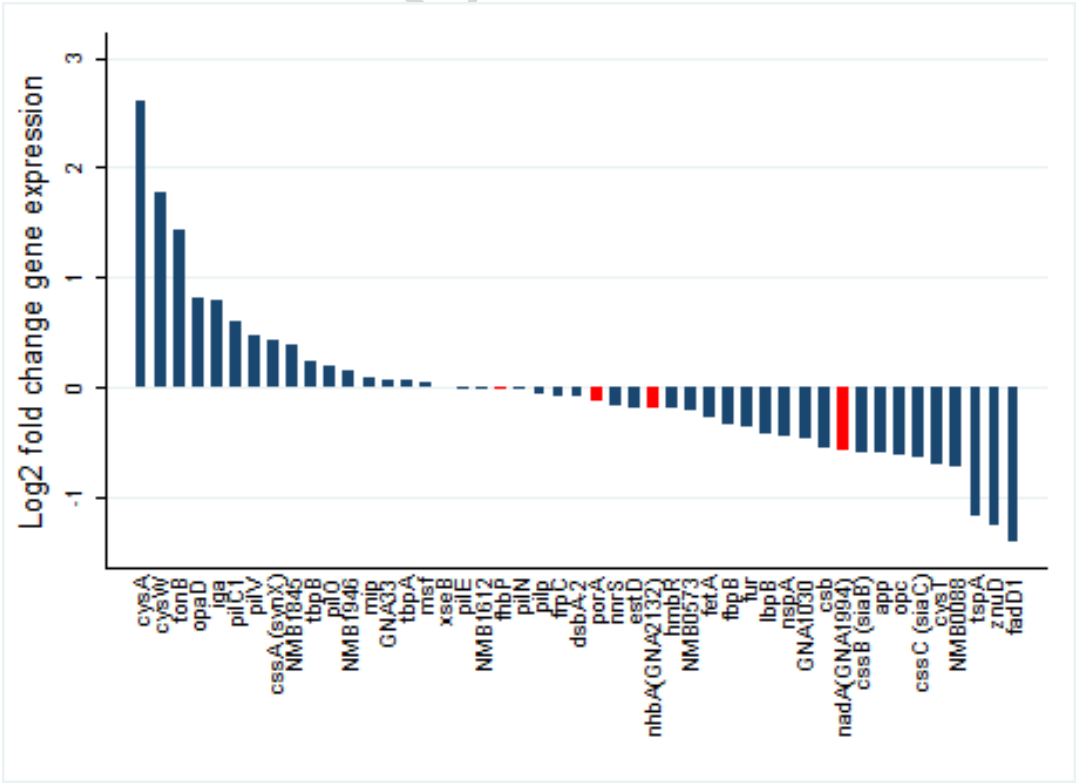
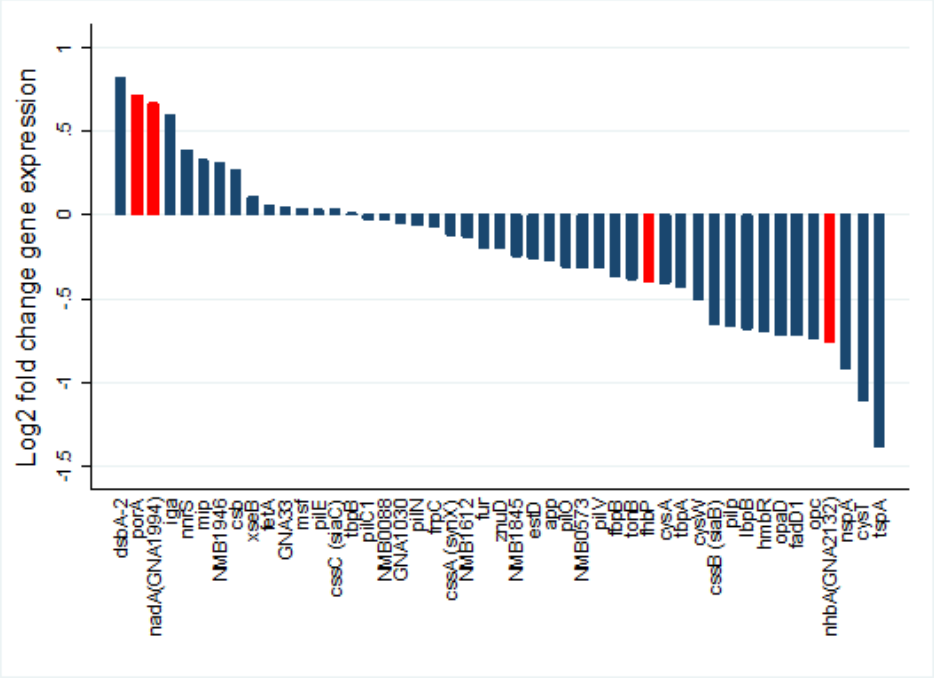


Figure 1. Mean gene counts of *Neisseria meningitidis* for technical replicates 1 and 2 from 100ng/µl total RNA.

A wide range of expression of the 47 gene transcripts (up to 2.7-fold, Log2 fold changes) was observed after exposure to low and high temperatures vs. 37°C (Figure 2).



(A)



(B)

Figure 2: Log2 fold change in gene expression across a panel of 47 genes showing up/down regulation of *Neisseria meningitidis* genes after (A) incubation at 26°C and (B) incubation at 40°C. Vaccine genes are shown in red.

After cold shock *cysA, cysW* and *tonB* showed the highest level of up-regulation, while after heat shock, *porA, dsbA-2* and *nadA* showed most up-regulation. For the vaccine genes, there was demonstrable down-regulation of *nadA* after cold shock, whereas after heat shock *porA* and *nadA* were upregulated and *fhbP* and *nhbA* were down-regulated (Table 1).

Mean controls	Group		Mean difference	P value	[95% Conf.	Interval]
152732.3	**Cold shock vs Control**	*porA*	-13245.0	0.4	-44151.3	17661.2
51152.9		*fhbP*	-736.8	0.7	-4087.6	2614.1
3440.4		*nadA*	-404.3	0.003	-669.78	-138.9
1218.6		*nhbA*	-454.1	0.1	-991.29	83.1
	Heat shock vs Control					
152732.3		*porA*	96803.4	0.001	65897.2	127709.7
51152.9		*fhbP*	-12401.9	0.001	-15752.8	-9051.2
3440.4		*nadA*	712.0	0.001	446.6	972.4
1218.6		*nhbA*	-1416.8	0.001	-1954.0	-879.7

Table 1: Fold change in expression of Neisseria meningitidis genes encoding meningococcal vaccine proteins after incubation at 26°C (cold shock) and 40°C (heat shock) compared to incubation at 37°C.

Guckenberger et al (14), who used micro-arrays to measure Nm gene expression after heat shock at 45°C, had results consistent with those in our study, including for example, up-regulation of *dsbA-2* and down-regulation of *pilO* and genes involved in the ABC transport system (*cysA, cysW, cysT* in our panel). Interestingly, in our study, two of these genes (*cysA, cysW*) were up-regulated on cold exposure.

This pilot study of gene expression profiling using a digital multiplexed gene detection system showed that mRNA can be detected and quantified from Nm cultures. The objective of this study was to assess the method (sensitivity, reproducibility, ability to detect fold change). The NanoString technique was highly reproducible, and we were able to detect transcripts from a very low quantity of total RNA. Application of this technique to Nm pharyngeal samples will be challenging as RNA concentrations in such samples will be relatively low, variation in levels of gene expression is wide, and RNA from other pharyngeal bacteria is likely to be present. For multi-copy genes, as our probes were targeted at conserved regions, a limitation is that we were unable to identify which copies were expressed, so these results should be interpreted with caution. Although the reliability of the NanoString platform has been extensively reported for other organisms (3-6, 15) , we plan to cross-validate our results with Nm with other methods such as reverse transcriptase qPCR for selected genes in subsequent experiments.

Acknowledgements

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Conflict of interest

HC reports receiving honoraria from Sanofi Pasteur, and consultancy fees from AstraZeneca, IMS Health and GSK, all paid to her employer. AF is the chief investigator on grants awarded to University of Bristol from GSK and Pfizer who manufacture meningococcal vaccines.

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Highlights

- *N. meningitidis* gene transcripts were quantified using NanoString technology for the first time.
- The method was sensitive and reproducible.
- Fold changes in gene expression were demonstrated after thermal stress.